The DNA Polymerase Gamma R953C Mutant Is Associated with Antiretroviral Therapy-Induced Mitochondrial Toxicity

Min Li, Andrea C. Mislak, Yram Foli, Esinam Agbosu, Vivek Bose, Shreya Bhandari, Michal R. Szymanski, Christie K. Shumate, Y. Whitney Yin, Karen S. Anderson, Elijah Paintsil

Department of Pediatrics, Yale School of Medicine, New Haven, Connecticut, USA; Department of Pharmacology, Yale School of Medicine, New Haven, Connecticut, USA; Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas, USA; Department of Epidemiology & Public Health, Yale School of Medicine, New Haven, Connecticut, USA

We found a heterozygous C2857T mutation (R953C) in polymerase gamma (Pol-γ) in an HIV-infected patient with mitochondrial toxicity. The R953C Pol-γ mutant binding affinity for dCTP is 8-fold less than that of the wild type. The R953C mutant shows a 4-fold decrease in discrimination of analog nucleotides relative to the wild type. R953 is located on the “O-helix” that forms the substrate deoxynucleoside triphosphate (dNTP) binding site; the interactions of R953 with E1056 and Y986 may stabilize the O-helix and affect polymerase activity.

Antiretroviral therapy (ART)-related toxicities predominantly manifest in mitochondrial dysfunction. A critical backbone of ART is nucleoside reverse transcriptase inhibitors (NRTIs). With widespread use of NRTIs, clinical manifestations such as lactic acidosis, lipodystrophy, peripheral neuropathies, cardiomyopathies, and pancytopenia were observed (1–3). These adverse effects of NRTIs were attributed to inhibition of the polymerase gamma enzyme (Pol-γ), responsible for mitochondrial DNA (mtDNA) replication (4). The role of mutant Pol-γ variants in ART-related toxicity has not been systematically investigated. Only two Pol-γ mutations (R964C and E1143G) have been associated with ART-induced mitochondrial toxicity (5, 6).

We hypothesized that Pol-γ mutations might predispose patients toward developing mitochondrial toxicity. We performed a retrospective analysis of data and specimens collected during a prospective, case-control study of ART-induced mitochondrial toxicity (i) to investigate whether Pol-γ mutations are associated with ART-induced mitochondrial toxicity and (ii) to characterize the biochemical effect of these mutations, if any, on Pol-γ activity. The details of the study design have been previously published (7, 8). In brief, the cases comprised HIV-infected individuals identified by their HIV care providers as having symptoms consistent with ART-induced mitochondrial toxicity (2, 9). The study protocol was approved by the Institutional Review Board of the Yale School of Medicine. All participants gave their written informed consent before participation in the study.

The study included 45 African Americans (15 HIV-infected individuals with mitochondrial toxicity [9], cases; 15 HIV-infected individuals without toxicity, positive controls; and 15 HIV-uninfected individuals, negative controls). The demographic and clinical characteristics of participants are illustrated in Table S1 in the supplemental material. We amplified and sequenced the entire POLG genome, comprising 22 exons, of the 45 study participants using 16 pairs of overlapping primers (see Table S2) and a previously described PCR protocol (10). We observed a heterozygous C2857T mutation in exon 18 (see Fig. S1 in the supplemental material) of the POLG catalytic active site, corresponding to a substitution of R953 in the wild type (WT) to cysteine, yielding mutant R953C (Fig. 1A), in one HIV-infected patient with mitochondrial toxicity and observed no mutations in the two control groups. The catalytic site of Pol-γ is highly conserved among species (Fig. 1B), and mutations in this area could lead to depletion of mtDNA and are associated with mitochondrial diseases (6). Therefore, we investigated the mtDNA copy number of the patient with R953C relative those of the two controls. Fragments of the mitochondrial D loop and 18S rRNA nuclear gene were amplified using quantitative reverse transcription-PCR (RT-PCR) and primers (11, 12). The case patient with the R953C mutation had a significantly reduced mtDNA copy number compared with the positive control (P = 0.001) and negative control (P < 0.001) (Fig. 1C).

Given its location in the catalytic active site, we sought to determine the dissociation constant (K_d) of R953C and WT Pol-γ for the DNA primer-template substrate. Electrophoretic mobility shift assay (EMSA) analysis showed that the R953C Pol-γ binds DNA with an affinity that is not significantly different from that of WT Pol-γ (11.4 ± 0.6 nM and 18.4 ± 0.8 nM, respectively; data not shown) and is consistent with the low nanomolar affinities previously reported for WT Pol-γ (13, 14). To understand the molecular mechanism of the contribution of the Pol-γ R953C mutant to ART-induced toxicity, we employed a pre-steady-state kinetic approach as previously described (13, 14). Since the patient with the Pol-γ R953C mutation had been on lamivudine (3TC) for 10 years prior to diagnosis, we examined incorporation of the natural nucleotide dCTP into DNA by Pol-γ in vitro in the presence of WT or R953C mutants. The dissociation constant (K_d) of wild type (WT) Pol-γ for dCTP is 0.06 nM, and the dissociation constant (K_d) for the mutant R953C is 8-fold higher (0.6 nM). Thus, the incorporation of dCTP by Pol-γ is reduced.

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Address correspondence to Y. Whitney Yin, yinyin@yimodom, Karen S. Anderson, karen.anderson@yale.edu, or Elijah Paintsil, elijah.paintsil@yale.edu.

ML and A.C.M. contributed equally to this article.

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relative to the active triphosphate form of 3TC [(−)-3TC-TP]. A series of pre-steady-state burst and single-enzyme-turnover experiments were carried out with WT and mutant R953C Pol-γ/Pol-γ holoenzyme, evaluating single-nucleotide incorporation to determine the binding affinity ($K_d$), maximum incorporation ($k_{pol}$), and incorporation efficiency ($k_{pol}/K_d$) for dCTP and (−)-3TC-TP. The R953C Pol-γ $K_d$ for dCTP was 8-fold lower than that of the WT (Table 1), and the $k_{pol}$ for dCTP incorporation by R953C Pol-γ into a growing DNA chain was about 2 times higher than that of the WT, resulting in a 3.6-fold decrease in the efficiency of dCTP incorporation. We also observed a similar reduction in the incorporation efficiency for another natural nucleotide substrate, dTTP (data not shown).

On the other hand, the binding affinity of R953C Pol-γ for (−)-3TC-TP was slightly higher than that of the WT whereas the rates of incorporation were the same for the mutant and the WT. This resulted in a 4-fold reduction in the ability of the R953C Pol-γ mutant to discriminate between (−)-3TC-TP and dCTP (Fig. 2A).

To explain the decreased ability of the R953C Pol-γ mutant to discriminate between (−)-3TC-TP and dCTP, we modeled the relationship of deoxynucleoside triphosphate (dNTP) with the side chain of R953C based on the solved crystal structure of the human

<table>
<thead>
<tr>
<th>Pol-γ variant</th>
<th>Nucleotide</th>
<th>$K_d$ (µM)</th>
<th>$k_{pol}$ (s⁻¹)</th>
<th>Efficiency ($E_{natural,dNTP}/E_{analog}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>dCTP</td>
<td>1.3 ± 0.2</td>
<td>67 ± 3</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>(−)-3TC-TP</td>
<td>13 ± 5</td>
<td>0.12</td>
<td>0.009</td>
</tr>
<tr>
<td>R953C</td>
<td>dCTP</td>
<td>11 ± 2</td>
<td>150 ± 10</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>(−)-3TC-TP</td>
<td>11 ± 5</td>
<td>0.12</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* NRTI, nucleoside reverse transcriptase inhibitor; 3TC, lamivudine; TP, triphosphate; Pol-γ, polymerase gamma; $k_{pol}$, maximum rate of incorporation; $K_d$, equilibrium dissociation constant; $E_{natural,dNTP}/E_{analog}$, efficiency ratio for dCTP/(−)-3TC-TP.
the homozgyous Pol-γ R953C mutation has been linked to PEO, while heterozygous family members were asymptomatic (10, 17). Additionally, a molecular analysis of POLG in 2,697 patients identified the R953C mutation in trans with the W748S mutation in a patient with moderate depletion of mtDNA levels (61% of the level seen with age-matched controls) (18). Another study of 92 patients with POLG R953C in trans with W748S showed the development of mitochondrial neurogastrointestinal encephalopathy (MNGIE)-like disorder, an autosomal recessive disorder characterized by severe gastrointestinal dysmotility, cachexia, PEO and/or ptosis, and peripheral neuropathy (19). Functional genetic variants of POLG can be as seen at rates as high as 2% (5), and an aggregate study of 121,421 chromosome samples identified the R953C mutation in 0.001647% (20).

The R964C Pol-γ homozgyous mutant was first identified in an HIV-infected individual on NRTI-based ART (3TC and stavudine [d4T]) who developed severe lactic acidosis (5). A detailed pre-steady-state kinetic analysis showed that R964C had a decrease in dTTP incorporation efficiency compared with the wild-type Pol-γ as well as lower dTTP-TP discrimination (21), indicating that the R964C Pol-γ mutant predisposes patients to ART-induced mitochondrial toxicity. The second Pol-γ mutation reported to be associated with ART toxicity, E1143G/D, was found in 10 of 69 HIV-infected patients with lipodystrophy (6). However, the Pol-γ E1143G mutant did not affect the activity of the enzyme (22, 23). Taking the data together, our finding is consistent with the concept of predisposition of Pol-γ mutations to ART-induced toxicity.

Our study had several limitations. First, the diagnosis of mitochondrial toxicity was not confirmed with a tissue biopsy specimen; therefore, some of the clinical symptoms of the cases could have been misclassified. Second, the small sample size limits the generalization of our finding. The reported prevalence of the R953C mutation with ART-induced toxicity was 0.001647% (20); thus, our finding is intriguing and warrants longitudinal (e.g., pre- and post-ART) studies to validate our observations. The strengths of our study were that we had epidemiologic, biochemistry, and mitochondrial biogenesis data in support of our finding.

In conclusion, we report a novel association of Pol-γ R953C mutation with ART-induced toxicity. The Pol-γ mutation was associated with decreased enzyme activity and mtDNA content. On the basis of our finding and previously published data, we hypothesize that Pol-γ mutations and/or polymorphisms might predispose patients to ART-induced mitochondrial toxicity. Studies to investigate Pol-γ mutations that could predispose patients to ART toxicity might inform the selection of appropriate patient-specific NRTIs to avoid ART-induced toxicity.

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We do not have a commercial or other association that might pose a conflict of interest.

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